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Research article

Sustainable extracts from oliviculture by-products: Phenolic content and its antimicrobial activity against foodborne pathogens

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Abstract: Olive leaves and two-phase olive pomace (alperujo) are abundant agro-industrial by-products with potential as sustainable sources of bioactive compounds. In this study, aqueous and 70% ethanolic extracts obtained from leaves of the Ascolano and Grappolo cultivars and from alperujo were characterized for their phenolic composition and evaluated for antimicrobial activity against foodborne pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Salmonella* Enteritidis. Phenolic compounds were analyzed by HPLC-UV/Vis, and antimicrobial activity was assessed by broth microdilution and minimum bactericidal concentration assays. In general, ethanolic extracts showed broader phenolic profiles and stronger antimicrobial effects than aqueous extracts, indicating more efficient recovery of active compounds by hydroalcoholic extraction. The ethanolic extract of Grappolo leaves showed the highest antibacterial activity, achieving > 90% inhibition against *B. cereus*, *E. coli*, and *S. aureus*, and was the only extract to exhibit bactericidal activity at 50% against these microorganisms. This extract was also characterized by high hydroxytyrosol content, whereas the ethanolic alperujo extract showed the highest oleuropein concentration. The stronger activity of ethanolic extracts is likely related to their greater ability to solubilize phenolic compounds with antimicrobial relevance, particularly hydroxytyrosol and oleuropein, which may act through membrane disruption, interference with permeability, and impairment of essential cellular functions. Overall, the results support olive leaves and alperujo as promising low-value raw materials for recovering phenolic-rich extracts with potential

applications as natural antimicrobial agents in food systems. To improve reproducibility and practical applicability, standardization of extract yield and concentration should be considered in future studies.

Keywords: antimicrobial activity; food additives; olive leaves; residue; sustainability

1. Introduction

Olive trees, *Olea europaea L.*, belong to the botanical family Oleaceae and are native to Mediterranean countries; therefore, this region is the largest producer of olives and olive oil, accounting for 98% of the world's olive tree production. Olive tree products are rich in important substances from a nutritional perspective since they have antioxidant and antimicrobial properties attributed to their phenolic compounds, particularly hydroxytyrosol, and oleuropein [1].

Olive by-products, often referred to as residues or waste products, are derived from the cultivation and processing of olive trees and olive oil, and are produced annually in large quantities [2]. Unfortunately, most of these by-products do not have practical applications and go to waste; the use of both can be the focus of a sustainable valuation of new products [3]. One of the most abundant by-products of growing olives is olive leaves, which have antioxidant, anti-inflammatory, antimicrobial, and antiviral activity, and can be considered a cheap and natural source of antioxidants [4–6].

The recovery of phenolic compounds from olive by-products has gained attention as part of sustainable and circular bioeconomy strategies. Studies have highlighted the relevance of olive pomace valorization and phenolic characterization, as well as the potential antimicrobial application of these recovered compounds [7,8]. In addition, there are residues from processing olives to extract olive oil, which represent a huge environmental problem, as they are produced in large quantities and have a high content of organic matter, such as polyphenols, which may be responsible for high toxicity [9]. Therefore, the use of this residue requires a significant economic effort to avoid severe environmental pollution, and it can be used as an economic source with antimicrobial and antioxidant activity to prevent environmental damage arising from its misdirection [10].

Usually, for the production of olive oil, the centrifugation method is used, which can be a continuous three-phase process, where the waste produced comprises the liquid wastewater from the olive mill wastewater, and bagasse, which corresponds to solid waste, also known as Alperujo [11]. The process can also be carried out in a two-phase system for the production of olive oil, resulting in a solid two-phase olive oil extraction residue (alperujo), which consists of a thick mass made up of the pulp and olive stone, as well as the water from the fruit and residual oil [12].

Due to the increased resistance of pathogenic microorganisms to numerous drugs and the susceptibility of these drugs to various substrates, there is concern about the need to search for new alternatives, which encourages the search for natural antibiotics. According to the World Health Organization (WHO), foodborne infections remain one of the leading causes of disease worldwide [13].

Various approaches have been used to address contamination by pathogenic microorganisms, such as the use of preservatives. In this context, due to concerns about the toxicity and dangers of synthetic preservatives, the demand for natural food preservatives has increased, and plant extracts emerge as a better option, as they are rich in bioactive compounds that act as natural preservatives [14]. Olive-growing by-products, being natural compounds, can serve as an alternative source of antimicrobials, potentially replacing chemical preservatives, which may be toxic and carcinogenic.

Several studies have demonstrated the antimicrobial activity of olive leaf extracts against foodborne and clinically relevant microorganisms. Compounds such as oleuropein, hydroxytyrosol, luteolin derivatives, and verbascoside have been associated with inhibitory effects against *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* spp [5,6,15]. Despite these advances, most researchers focus exclusively on olive leaves, while fewer researchers evaluate residues from olive oil production as potential antimicrobial sources. Furthermore, the antimicrobial activity of olive-derived extracts is strongly influenced by cultivar, extraction solvent, and phenolic composition, making direct comparisons between studies difficult [6,16]. Limited information is available on the comparative antimicrobial potential of olive cultivars and olive by-products analyzed under the same experimental conditions, particularly against foodborne pathogens of public health importance.

Given the above, we aimed to characterize extracts from olive leaves (*Olea europaea*) and alperujo residues from olive oil production in a two-phase system, with respect to phenolic content and antimicrobial activity against food-contaminating bacteria.

2. Materials and methods

2.1. Collection and sampling locations

The alperujo *residues* of the *olive oil* production and the olive leaves used to manufacture the extracts were kindly provided by the Experimental Farm of EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais) in Maria da Fé, Brazil (22◦18' south latitude, 45◦23' west longitude, an average elevation of 1.276 m). Olive leaves were randomly and separately picked from olive branches of the Ascolano and Grappolo 546 cultivars. Approximately 1 kg of the alperujo *residue* of the *olive oil* production in a two-phase system (solid fraction) from unripe olives (green olives) containing olive pulp, skin, stone, water, and some concentration of olive oil, was collected.

All materials were collected in sterile plastic bags and kept refrigerated to the Laboratory of Microbiology of Fermentations, in the Microbiology sector, Department of Biology, Federal University of Lavras (UFLA), Lavras, Brazil, for the preparation of extracts.

2.2. Preparation of extracts

Two extraction methods were used, including one using water and another using 70% ethanol. Olive leaves were washed with deionized water and dried at 37 ℃ for 3 days, and the alperujo residue was directly placed in an oven for drying under the same conditions. Then the dried leaves and alperujo residue were pulverized in a conventional blender to reduce the particle size to 90–150 μm. For each extraction method, 3 different extracts were prepared: Olive leaves Ascolano extract, olive leaves Grappolo extract, and the residues of the olive oil production extract.

2.2.1. Aqueous extract

The methodology described by Al-Attar and Abu Zeid [17] was used with some modifications. A total of 50 g of powder made from dried olive leaves and alperujo residue were added to 2 L of hot water. After 3 h, the mixture was slowly boiled for 30 min. After boiling, the mixture was cooled to room temperature and then processed in an electric mixer for 20 min. Subsequently, the solutions were centrifuged (5 min at 5000 rpm) and filtered through a sterile syringe filter (0.2 μm, PES). The filtrate was evaporated using on a rotary evaporator at 40 ℃ to obtain the dry residue (active ingredients) and

stored at 4 ℃ for use throughout the experiment.

2.2.2. Ethanol extract

The extracts were obtained using the methodology proposed by LingYapRadhakrishnan et al. [18] with some modifications. Dried olive leaves and alperujo residue (50 g) were added with 750 ml of ethanol (70% v/v). The extraction was performed at room temperature, and the samples were left to rest for 24 h, so the extracts were obtained after 24 h. The extracts were then centrifuged (5 min at 5000 rpm) and filtered through a sterile syringe filter (0.2 µm, PES). Then, the solvent was removed using a rotary evaporator at 38 ℃ vacuum and 120 rpm under reduced pressure. The extracts were stored at 4 ℃ for use throughout the experiment.

Following solvent evaporation, the extracts were reconstituted in 10 mL of solvent. Therefore, the stock extracts corresponded to the extractable fraction obtained from 50 g of dried olive leaves or alperujo residue in a final volume of 10 mL. Antimicrobial assays were performed using serial volumetric dilutions of these reconstituted extracts.

2.3. Tested microorganisms

The following bacteria were used: Gram-positive (*L. monocytogenes* ATCC 19117, *S. aureus* ATCC 8702, and *Bacillus cereus* ATCC 14579) and Gram-negative bacteria (*E. coli* EPEC055 and *S,* Enteritidis ATCC 564). The inocula were standardized with the aid of a growth curve, following the absorbance (O.D. 600nm) and the plate count using Brain Heart Infusion (BHI) agar (Sigma Aldrich, Darmstadt, Germany). The plates were incubated at 37 ℃ for 24 h, and the inoculum was standardized at 108 CFU. mL^{-1}. The inocula were stored in a freezer at –70 ℃ and thawed at room temperature during the experiment.

2.4. Antimicrobial activity

To determine the percentage of inhibition, the protocol established by CLSI [19] was used, with some adaptations, using the broth microdilution technique (sterile 96-well microplates). The culture medium used was BHI broth (Sigma Aldrich, Darmstadt, Germany).

To each well, 100 µL of BHI broth was added. In the first well of each line of microplates, a volume of 100 µL of the respective extract (resuspended in sterile water) to be analyzed was also added, obtaining the initial concentration of each extract (50%). From the initial wells, serial dilutions were performed, obtaining decreasing concentrations of 50%, 25%, 12.5%, and 6.25% (v/v). Aliquots of 10 µL of each bacterial suspension were inoculated into wells containing BHI medium and the respective concentration of extracts. Positive controls (PC) consisted of 10 µL of each bacterial suspension inoculated in BHI medium without the addition of extracts, and negative controls (NC) consisted of BHI medium supplemented with 10 µL of the different extract concentrations without the addition of bacterial suspension. The microplates were sealed and incubated in a BOD incubator at 37 ℃ for 24 h. All antimicrobial assays were performed in triplicate wells and repeated in three independent experiments. Results are presented as mean ± standard deviation.

After incubation, absorbance at 600 nm was measured using a UV spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the value of the percentage of inhibition was expressed as Eq (1):

$$Inhibition \ \ (\%) = \left[1 - \left(Ac - \frac{NC}{PC}\right)\right] x \ \ 100 \quad (1)$$

where Ac represents the absorbance of the well with different concentrations of extracts, NC is the negative control, and PC is the absorbance of the positive control [20].

2.5. Minimal bactericidal concentration (MBC)

After the incubation period was determined for the minimum bactericidal concentration (MBC), the micro drop technique was used with plating on BHI agar, and 10 µL aliquots of cultures from the wells were plated where there was no turbidity; these were put on the plates and incubated at 37 ℃ for 24 h. After incubation, the MBC of the extracts was determined, with the lowest concentration capable of promoting the absence of growth of the bacteria tested on the plate [21]. The experiment was carried out in triplicate and with three repetitions.

2.6. Identification of phenolic compounds contained in each extract by HPLC (UV-vis)

High-pressure liquid chromatography (HPLC) equipped with UV-visible absorption (UV-vis) was used for the analytical qualification and quantification of phenolic compounds in each olive leaf extract. The extracts (10 mg) were dissolved in 1 mL of 100 mM perchloric acid (Sigma Aldrich, Darmstadt, Germany) solution and filtered with 0.45µm filters into vials before analysis by HPLC (UV-vis). The separation of phenolic compounds was carried out in Shimpack SCR-101H column (7.9 mm × 30 cm) Shimadzu, the mobile phase was 100mM perchloric acid (Sigma Aldrich, Darmstadt, Germany) solution with a flow rate of 0.6mL/min over 20 min run, the oven temperature was 50°C, and a 210nm UV detector (30°C) was used for detection.

All phenolic compounds were identified by comparing their retention times with the respective standards (chlorogenic acid, caffeic acid, coumaric acid, hydroxytyrosol, oleuropein, and rutin) (Merck, Darmstadt, Germany), as shown in Figure 1. Before running the experiments, calibration curves were established for each analyte so that relative response factors were accounted for when reporting analyte concentrations. Analyses were performed in triplicate.

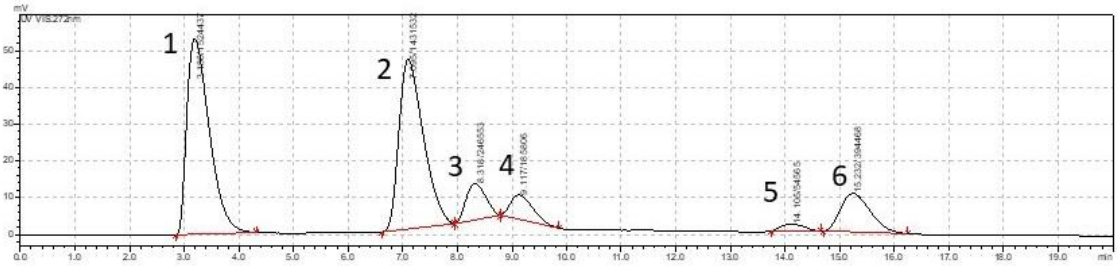

Figure 1. HPLC chromatograms of the standards. 1: Hydroxytyrosol; 2: Chlorogenic acid; 3: Rutin; 4: Caffeic acid; 5: Coumaric acid; and 6: Oleuropein.

2.6.1. Calibration curves

The calibration of the phenolic compounds method was conducted with 7 levels for chlorogenic acid, caffeic acid, coumaric acid, hydroxytyrosol, oleuropein, and rutin. The fit of the applied linear regression model was described as R^2 (> 0.99). Each set was made by dissolving the given mass in nanopure water to obtain L7. Levels 6-1 were then in the same-size volumetric flask used to prepare L7.

Each level was syringe filtered through a 0.45 µm nylon filter. Calibration curves were obtained from the area of each standard peak at each level. Each compound was identified by retention time (min).

2.7. Statistical analysis

The results were analyzed using GraphPad Prism version 8.01 for Windows (La Jolla, CA, USA). The two-way ANOVA and Tukey's multiple comparisons test were performed, and the significant differences were reported as: **** for $p<0.0001$; *** for $p<0.001$; ** for $p<0.01$; and * for $p<0.05$. Results were expressed as mean values ± standard deviation (n = 3).

3. Results and discussion

3.1. Identification of phenolic compounds

To the best of our knowledge, this is the first time that Ascolano and Grappolo olive leaves have been studied. Table 1 shows the phenolic composition of the extracts used in this study; the compounds identified were chlorogenic acid, caffeic acid, coumaric acid, hydroxytyrosol, oleuropein, and rutin. In the aqueous extracts, the compounds caffeic acid, coumaric acid, and oleuropein were not present in olive leaf extracts; in the aqueous extracts of olive oil production residues, caffeic acid was the only compound that was not present. The use of 70% ethanol (v/v) yielded higher concentrations and a greater variety of compounds in the extracts; therefore, ethanol was the solvent with the highest extraction capacity, which contributed to better antimicrobial activity. This fact agrees with the work of Ortega-Garc áBlancoPeinado et al. [22], which showed that the use of water combined with alcohols contributes to increased swelling of plant materials, resulting in the increased contact area between plant matrix and solvent, improving extraction yield and higher concentration of compounds.

Table 1. Phenolic compounds in extracts derived from olive leaves and alperujo residue of olive oil production ($mg.mL^{-1}$).

Extracts	Solvent	Hydroxytyrosol	Chlorogenic acid	Rutin	Caffeic acid	Coumaric acid	Oleuropein
EA	Aqueous	0.65 ± 0.08 ^{d}	0.076 ± 0.005 ^{c}	0.023 ± 0.003 ^{b}	-	-	-
EG		1.82 ± 0.13 ^{c}	0.076 ± 0.002 ^{c}	0.024 ± 0.001 ^{b}	-	-	-
ER		0.66 ± 0.11 ^{d}	0.072 ± 0.0006 ^{c}	0.019 ± 0.001 ^{b}	-	1.59 ± 0.09 ^{c}	0.79 ± 0.04 ^{c}
EA	Ethanolic	3.28 ± 0.46 ^{b}	0.087 ± 0.004 ^{b}	0.025 ± 0.001 ^{b}	0.89 ± 0.04^{c}	1.79 ± 0.09 ^{c}	1.83 ± 0.02 ^{b}
EG		9.24 ± 0.58 ^{a}	0.095 ± 0.003 ^{b}	0.033 ± 0.002 ^{a}	1.18 ± 0.04 ^{b}	2.75 ± 0.1 ^{b}	1.89 ± 0.04 ^{b}
ER		0.37 ± 0.07 ^{d}	0.72 ± 0.02 ^{a}	0.037 ± 0.003 ^{a}	1.48 ± 0.07 ^{a}	3.21 ± 0.28 ^{a}	16.47 ± 0.76 ^{a}

*Note: EA: Extract of Ascolano olive leaves; EG: Extract of Grappolo olive leaves; ER: Extract of the alperujo residue of olive oil production. -: not identified. The results are expressed as means. ± SD (n = 3). Values followed by different lowercase letters within the same column differ significantly according to Tukey's test ($p < 0.05$).

The compounds identified in this study have also been found in extracts of olive leaves from other species in other studies, including olive leaves collected in the southeast of Sicily, Italy in the work of Palmeri et al. [23]. Chlorogenic acid, hydroxytyrosol, and rutin were identified in the work by Zhang et al. [24] in olive leaves from cultivars grown in China; these compounds have remarkable antimicrobial properties [25]. Rutin was one of the flavonoids found in olive leaves in the work of

Pham et al. [26]. Caffeic acid, *p*-coumaric acid, and oleuropein were identified in ethyl alcohol extracts from olive leaves in the work of Koruk et al. [27].

Olive leaf extract and its constituents, particularly oleuropein and hydroxytyrosol, have been reported to have health benefits, including antioxidant and antimicrobial properties [28]. Normally, in olive leaf extracts, oleuropein is the compound present in the largest amounts [25]. On the other hand, in our study, the olive leaf extract of the two studied cultivars (Ascolano and Grappolo), hydroxytyrosol, was the compound present in higher (p<0.05) quantity, mainly in the Grappolo cultivar (9.24 ± 0.58 mg.ml^{-1}). Hydroxytyrosol is present in almost all parts of the olive tree, mostly in the leaves, as this compound is the main product of oleuropein degradation [29]. Therefore, the oleuropein present in large amounts in the extracts may have been converted into hydroxytyrosol, resulting in the high concentration of these compounds in the extracts.

Several studies have revealed various classes of phenolic compounds in olive residues from different olive tree varieties [30,31]. Normally, the phenolic compound oleuropein is the most abundant in olives from different cultivars [32]. Accordingly, our study showed a high concentration of oleuropein in olive oil production residues, ethanolic extracts (16.47 ± 0.76 mg/ml), which consists of a mass containing the pulp, pit, skin, and traces of olive oil. Ethyl acetate extracts of olive mill wastewater were studied in the work of Fki et al. [33]. Hydroxytyrosol was present at higher concentrations, whereas caffeic acid and coumaric acid were present at lower concentrations.

In the work of Dermeche et al. [34], the solid residue was analyzed; p-coumaric acid, caffeic acid, hydroxytyrosol, and rutin were identified in the extracts. The same was observed in our work; however, oleuropein was not present. On the other hand, this compound was present in higher (p<0.05) amounts in the extracts of alperujo residue in our study. One explanation could be the difference in olive maturation; in our work, the olives used to manufacture olive oil were unripe olives (green olives), and in the work where the samples were collected in the final stage, ripe olives (black olives) were used, meaning oleuropein was degraded into elenolic acid and hydroxytyrosol [35].

The major phenolic compounds found in olive oil processing waste in the work of Medeiros et al. [10] were hydroxytyrosol, oleuropein, tyrosol, caffeic acid, P-coumaric acid, vanillic acid, catechol, and rutin. Greco et al. [7] found that olive pomace from a two-phase olive oil extraction system, using green olives of the Coratina variety, contained hydroxytyrosol (~77%), tyrosol (~18.5%), verbascoside (~4%), and oleuropein (~0.5%). Therefore, these compounds represent a natural source of antioxidants and antimicrobials and can be used in the food industry, thereby replacing chemical preservatives associated with undesirable effects on human health.

3.2. Antibacterial activity

There is a critical need to combat emerging pathogenic microorganisms using diverse chemical structures and innovative mechanisms. Therefore, the use of plant-based antimicrobial agents is important, and researchers are dedicating efforts to using natural compounds against pathogenic microorganisms to be used as preservatives in food or to develop new drugs, which can be a potential alternatives to synthetic compounds that cause harmful effects on human health [36]. This study was conducted to evaluate the antimicrobial activity of *L. monocytogenes* ATCC 19117, *S. aureus* ATCC 8702, *B. cereus* ATCC 14579, *E. coli* EPEC055, and *S.* Enteritidis ATCC 564 against extracts of by-products generated in the production of olive oil and olive leaves.

Figure 2 shows the antimicrobial activity against the Gram-positive Bacterium *L. monocytogenes*. The olive leaf ethanolic extracts showed higher antimicrobial activity (Figure 2B), with a percentages of inhibition of 91.20 ± 1.10 and 74.84 ± 4.43% for Grappolo and Ascolano leaf extracts,

respectively, for the higher tested concentration. The highest (p<0.05) antimicrobial activity for the aqueous extracts (Figure 2A) was observed for the Ascolano leaf extract (57.51 ± 6.62) at 50% concentration. Regarding inhibition by the alperujo residue of the olive oil production extract, inhibition ranged from 16.37 ± 2.83 to 36.45 ± 4.09% for the ethanolic extracts, and from 14.82 ± 2.66 to 41.93 ± 3.21% for the aqueous extracts.

According to Gökmen et al. [37], there was a positive effect of olive leaf extracts on *L. monocytogenes*, where the antimicrobial activity was evaluated using disk diffusion microdilution methods. On the other hand, in the work of Hussain et al. [36], the leaf extracts from *Olea ferruginea*, using the same solvents as in our work, had no effect on the growth of *L. monocytogenes*.

In the work of Liu et al. [38], when studying the effect of olive leaf extract against *L. monocytogenes*, the authors found that with the use of extracts, the motility and biofilm formation of the bacterium were decreased. It has been suggested that olive leaf extract has the potential to be used in the food industry as an antimicrobial to inhibit the growth of foodborne pathogens in foods, on processing equipment, or in packaging materials.

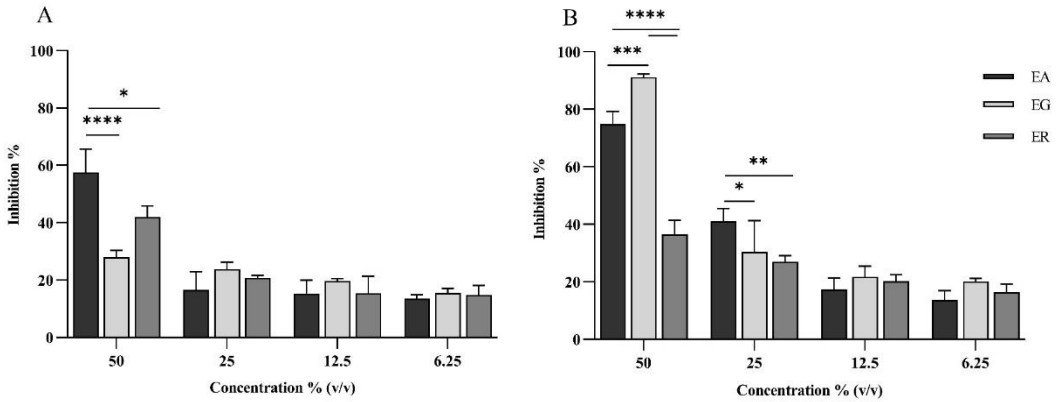

Figure 2. Percentage inhibition of *Listeria monocytogenes* by aqueous (A) and ethanolic (B) extracts obtained from olive leaves and alperujo residue. Values represent mean ± SD (n = 3). EA = Ascolano olive leaf extract; EG = Grappolo olive leaf extract; ER = alperujo extract. Statistical significance was determined by Tukey's multiple comparisons test (**** p<0.0001; *** p<0.001; ** p<0.01; and * p<0.05).

For the microorganism *B. cereus*, the results are presented in Figure 3. The ethanolic extracts (Figure 3B) showed high values of antimicrobial activity, with the highest (p<0.05) percentage found for the Grappolo leaf extract (92.24 ± 2.02%), followed by the alperujo residue (75.20 ± 3.62%), at the highest concentration tested. On the other hand, at a concentration of 50%, the aqueous extracts of Ascolano leaf exhibited higher (p<0.0001) antibacterial activity (46.71 ± 4.61%), followed by Grappolo leaf extract (27.51 ± 1.24%); this last one was the extract that presented higher (p<0.0001) inhibition at the lowest tested extract concentrations (25, 12.5, and 6.25%). The aqueous extract of *c* showed a low antimicrobial activity against *B. cereus*, with a maximum activity of approximately 8.85 ± 0.53% (Figure 3A).

B. cereus is a food-borne bacterium that is highly resistant and causes food poisoning. This organism is found throughout the world, thus making it a major concern for food safety [39]. In the work of PereiraFerreiraMarcelino et al. [40], olive leaf aqueous extracts (*Olea europaea* L. Cv. Cobrançosa) were screened for their antimicrobial activity, and *B. cereus* was the most sensitive

microorganism. Oleuropein, the main phenolic compound present in the alperujo residue (Table 1), can inhibit *B. cereus* sporulation [41]. Therefore, the extracts can be used to inhibit the growth of vegetative cells and to inhibit sporulation.

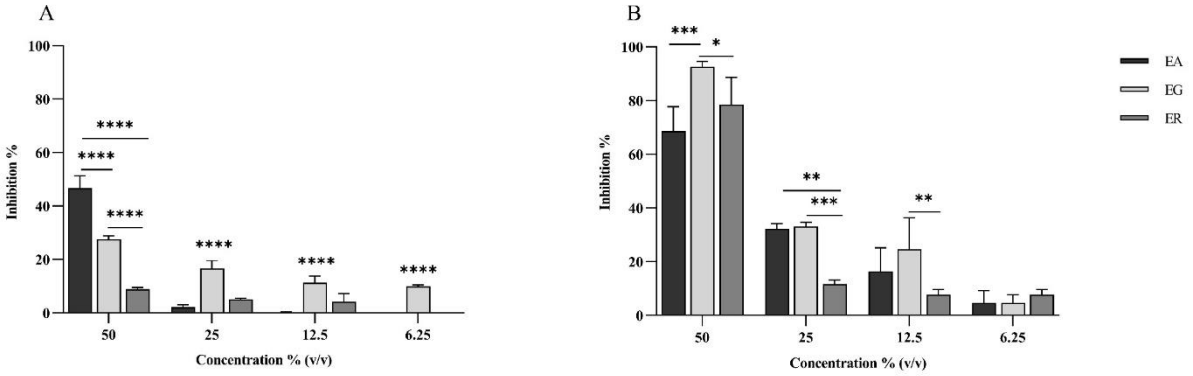

Figure 3. Percentage inhibition of *Bacillus cereus* by aqueous (A) and ethanolic (B) extracts obtained from olive leaves and alperujo residue. Values represent mean ± SD (n = 3). EA = Ascolano olive leaf extract; EG = Grappolo olive leaf extract; ER = alperujo extract. Statistical significance was determined by Tukey's multiple comparisons test (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; and * $p<0.05$).

The inhibition of the extracts against *S.* Enteritidis is shown in Figure 4. Of the aqueous extracts (Figure 4A), those from the Grappolo leaf showed the highest ($p<0.05$) inhibition, ranging from 12.61 ± 2.64% (6.25% concentration) to 41.14 ± 2.66% (50% concentration). The ethanolic extracts (Figure 4B) showed excellent antibacterial activities, with emphasis on the Grappolo leaf extract, which showed the highest ($p<0.0001$) inhibition at all concentrations tested, ranging from 41.47 ± 0.25% (for the lowest concentration of 6.25%) to 85.13 ± 4.61% (at the highest concentration of 50%). The antimicrobial activity of the other ethanolic extracts was similar ($p>0.05$) when tested at concentrations of 50, 25, and 6.25%, with an inhibition of 55.22 ± 4.37 for the Ascolano leaf extract, and 51.20 ± 2.97 for the aqueous extract, at the highest concentration. The inhibition *S.* Enteritidis, *L. monocytogenes*, and *E. coli* by olive leaf extract was confirmed in the work of Liu [15].

In most cases, gram-positive bacteria are more sensitive to natural plant compounds than gram-negative bacteria [42]. This is thought to be due to differences in the cell structure of these bacteria. In gram-positive bacteria, antibacterial substances can enter through the cell wall and attack the cytoplasmic membrane, resulting in cytoplasmic leakage [43]. Gram-negative bacteria, on the other hand, have an outer lipopolysaccharide component membrane that protects them from various agents [44]. However, in our work, we observed that the extracts showed high antimicrobial activity against the gram-negative *S.* Enteritidis bacterium, with an emphasis on the antimicrobial activity at low extract concentrations; the antimicrobial compounds present in the extracts likely have hydrophobic characteristics, which may contribute to the activity against Gram-negative bacteria. The activity against *S.* Enteritidis of oleuropein and caffeic acid was confirmed in the work of Lee and Lee [45].

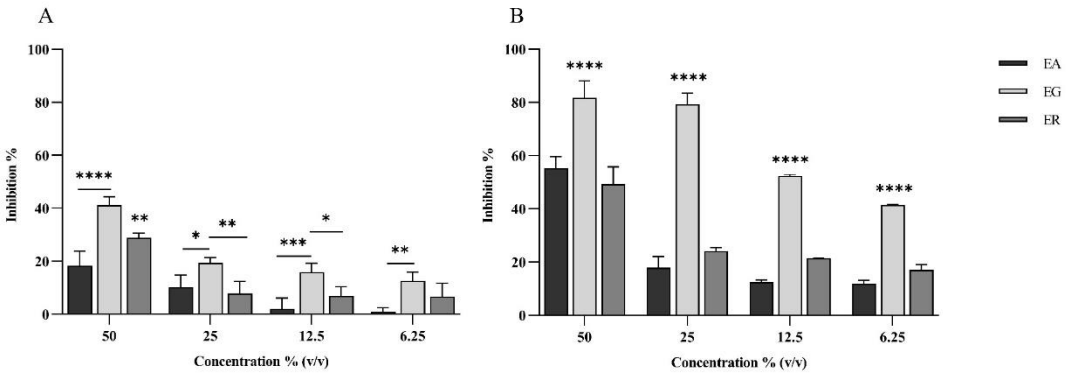

Figure 4. Percentage inhibition of *Salmonella* Enteritidis by aqueous (A) and ethanolic (B) extracts obtained from olive leaves and alperujo residue. Values represent mean ± SD (n = 3). EA = Ascolano olive leaf extract; EG = Grappolo olive leaf extract; ER = alperujo extract. Statistical significance was determined by Tukey's multiple comparisons test (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; and * $p<0.05$).

Figure 5 shows the percentage inhibition of the extracts against *S. aureus*. The aqueous extracts (Figure 5A) of Grappolo leaf and alperujo residue extracts showed the highest ($p<0.001$) inhibition with values of 39.83 ± 2.50% and 42.56 ± 4.48%, respectively, at a concentration of 50%. However, at the lowest concentration tested (6.25%), only the Ascolano leaf extract was able to inhibit the *S. aureus* bacterium (17.33 ± 2.16%); on the contrary, in the same concentration, the Ascolano leaves ethanolic extract showed no inhibition capacity (Figure 5B).

The highest antimicrobial activity was demonstrated by the ethanolic extracts at a concentration tested of 50%. Again, Grappolo leaf extracts showed the highest ($p<0.0001$) inhibition capacity (91.95 ± 3.50%), followed by Ascolano leaf extracts (60.61 ± 2.98%). The ethanolic extracts of the alperujo residue showed an antimicrobial activity ranging from 13.25 ± 2.25 to 42.77 ± 1.40% for the lowest and highest concentrations tested, respectively.

Aqueous extract from the "Chemlali" olive leaf showed the maximum antimicrobial activity against *S. aureus* in the work of Debib and Boukhatem [46]. The ethanolic extract in the concentration of 100% of "Dathier" olive leaves showed the greatest inhibition potential against *S. aureus* [47]. Hydroxytyrosol and oleuropein are phenolic compounds that have been proven to be effective against several human intestinal or respiratory tract pathogens by inhibiting or delaying their growth rate, and different targets have been detected for *S. aureus* [28].

The effect of olive mill wastewater extract was tested on *S. aureus* and *E. coli* in the work of Abu-Lafi et al. [48], who showed an inhibition zone equal to 23 and 25 mm, respectively, having a higher antibacterial activity compared to well-known antibiotics.

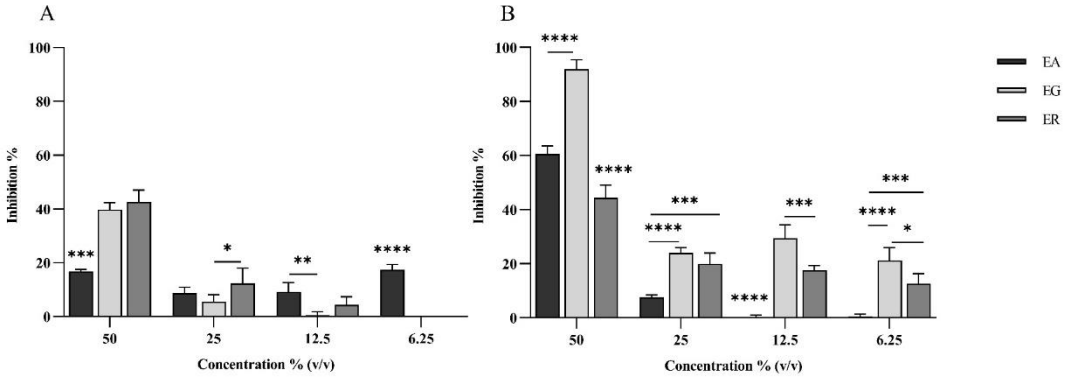

Figure 5. Percentage inhibition of *Staphylococcus aureus* by aqueous (A) and ethanolic (B) extracts obtained from olive leaves and alperujo residue. Values represent mean ± SD (n = 3). EA = Ascolano olive leaf extract; EG = Grappolo olive leaf extract; ER = alperujo extract. Statistical significance was determined by Tukey's multiple comparisons test (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; and * $p<0.05$).

The results of the antimicrobial activity against *E. coli* are shown in Figure 6. For the aqueous extracts (Figure 6A), the alperujo residue from olive oil production was the least efficient ($p<0.01$) at inhibiting the microorganism studied at a concentration of 50% (32.16 ± 3.59%). On the other hand, Ascolano and Grappolo leaf extracts showed similar inhibition values ($p>0.05$), with 44.07 ± 1.71% and 38.94 ± 4.99% inhibition, respectively. As observed for all studied microorganisms, Grappolo leaf ethanolic extracts were also more efficient ($p<0.0001$) in inhibiting *E. coli* (91.43 ± 0.35%), followed by Ascolano leaf ethanolic extracts (80.16 ± 2.17%) (Figure 6B).

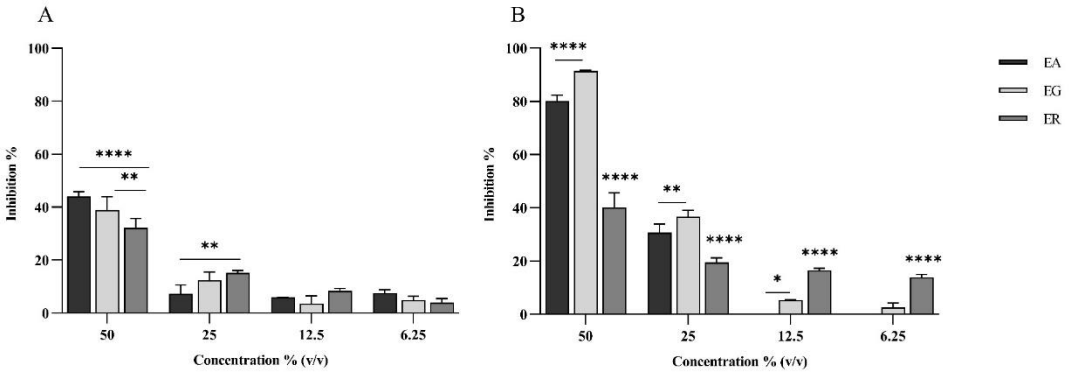

Figure 6. Percentage inhibition of *Escherichia coli* by aqueous (A) and ethanolic (B) extracts obtained from olive leaves and alperujo residue. Values represent mean ± SD (n = 3). EA = Ascolano olive leaf extract; EG = Grappolo olive leaf extract; ER = alperujo extract. Statistical significance was determined by Tukey's multiple comparisons test (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; and * $p<0.05$).

Extracts from olive mill residues (Mission and Frantoio olive fruit) were evaluated for phenolic compounds and their antimicrobial and antioxidant activity, resulting in broad-spectrum antibacterial activity against *S. aureus*, *Bacillus subtilis*, *E. coli*, and *Pseudomonas aeruginosa* [49].

The bacterium *E. coli* was sensitive to acetone, ethyl alcohol, and diethyl ether extracts from olive leaves in the work of Korukluoglu et al. [27]. However, the aqueous extract had no antimicrobial activity. In addition, the authors observed antimicrobial activity for *p*-coumaric acid, caffeic acid, and oleuropein against *E. coli*.

In Table 2, it is observed that only Grappolo leaf ethanolic extracts presented an MBC of 50% for the microorganisms *B. cereus*, *E. coli*, and *S. aureus*, while the bactericidal activity was not observed for the other microorganisms. The other extracts showed only inhibitory activity, and not being able to cause microbial death.

Table 2. Minimum bactericidal concentration (MBC) evaluated to extracts derived from olive leaves and alperujo residue of olive oil production.

Extract		Microorganisms				
		L. monocytogenes	*B. cereus*	*S.* Enteritidis	*S. aureus*	*E. coli*
EA	Aqueous	-	-	-	-	-
EG		-	-	-	-	-
ER		-	-	-	-	-
EA	Ethanolic	-	-	-	-	-
EG		-	50%	-	50%	50%
ER		-	-	-	-	-

*Note: EA: Extract of Ascolano olive leaves; EG: Extract of Grappolo olive leaves; ER: Extract of alperujo residue of olive oil production. (-): Bactericidal activity was not observed.

Again, we can observe that ethanol is a more effective extracting solvent when compared to water for the extracts of by-products generated in the production of olive oil and olive leaves. As such, ethanol showed great extraction efficiency; these results are in line with other authors [27,50–52]. The type of solvent affects the distribution and number of phenolic compounds present in the extracts, and consequently, the antimicrobial activity [27].

The higher antimicrobial activity observed for the ethanolic extracts may be related to their higher concentrations of hydroxytyrosol and oleuropein, supporting the hypothesis that these compounds contributed substantially to the inhibitory effects detected against the tested foodborne pathogens. It has been reported that hydroxytyrosol exerts antimicrobial effects through multiple mechanisms, including disruption of bacterial membrane integrity, alteration of membrane permeability, and induction of oxidative stress. These effects can lead to the leakage of intracellular components, impairment of essential metabolic processes, and, ultimately, cell death. In particular, hydroxytyrosol may interfere with biofilm formation and bacterial adhesion, further contributing to its antimicrobial efficacy [1,53].

Oleuropein has also been shown to possess broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria. It is believed that the antimicrobial action of oleuropein involves interactions with bacterial cell membranes, resulting in structural damage to the cell membranes and disruption of nutrient transport systems. In addition, oleuropein and its degradation products may interact with proteins and enzymes involved in bacterial metabolism, reducing microbial growth and

viability. Studies have suggested that oleuropein may also inhibit amino acid utilization and interfere with the production of enzymes necessary for bacterial replication [1,54].

Therefore, agro-industrial byproducts can contain high concentrations of bioactive molecules that, when extracted using environmentally friendly methods, may show promise in controlling microorganisms [8]. The antimicrobial activity observed in this study suggests that olive leaf and alperujo extracts could potentially be used in food systems frequently associated with the microorganisms evaluated, including ready-to-eat meat products, dairy products, fresh vegetables, minimally processed foods, and poultry products, which are commonly implicated in contamination by *L. monocytogenes*, *S. aureus*, *B. cereus*, *E. coli*, and *S.* Enteritidis [55,56].

Several researchers have already explored the incorporation of olive-derived extracts into food matrices and packaging materials [57–59]. Olive leaf extracts have been investigated as natural preservatives in meat and dairy products, fish, edible coatings, and active packaging systems, where they contributed to the inhibition of microbial growth and the extension of shelf life while providing antioxidant protection [60–63]. The high concentrations of hydroxytyrosol and oleuropein detected in this study further reinforce the potential technological value of these extracts, as both compounds are recognized for their antimicrobial and antioxidant properties.

From an industrial perspective, olive byproducts are an attractive raw material because they are abundant, inexpensive, and readily available in olive-producing regions. Their valorization aligns with the principles of the circular economy and can help reduce the environmental impact of olive oil production waste. However, before large-scale application can be considered, further studies are needed to evaluate the efficacy of these extracts directly in food systems, as interactions with food components may reduce antimicrobial efficacy. Additionally, sensory acceptance, potential effects on flavor, aroma, and color, toxicological safety, regulatory requirements, and economic viability must be carefully assessed. Although antimicrobial activity was assessed using volumetric dilutions of the reconstituted extracts (% v/v), future studies could benefit from additional standardization based on dry extract yield and should investigate the performance of these extracts under real-world food processing and storage conditions.

4. Conclusions

Our results of this study demonstrate that extracts obtained from olive leaves and the olive pomace generated during olive oil production, particularly the ethanolic extracts, are rich sources of phenolic compounds and exhibit antimicrobial activity against important foodborne pathogens. The higher antimicrobial activity observed in some extracts was associated with high concentrations of hydroxytyrosol and oleuropein, highlighting the potential of olive byproducts as value-added resources.

These findings support the valorization of olive industry by-products within a circular economy and suggest that such extracts may represent promising natural alternatives for food preservation and other biotechnological applications. However, further research is needed to evaluate their efficacy in real food matrices, determine optimal application concentrations, assess sensory impacts and consumer acceptance, and establish their safety and economic viability under industrial conditions. Such studies will be essential to support the future development of olive-derived antimicrobial ingredients for commercial applications.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors report there are no competing interests to declare.

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